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# Nonmitogenic Anti-CD3 Monoclonal Antibodies Deliver a Partial T Cell Receptor Signal and Induce Clonal Anergy

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## Summary

Anti-CD3 monoclonal antibodies (mAbs) are potent immunosuppressive agents used in clinical transplantation. However, the activation-related adverse side effects associated with these mAbs have prompted the development of less toxic nonmitogenic anti-CD3 mAb therapies. At present, the functional and biochemical consequences of T cell exposure to nonmitogenic anti-CD3 is unclear. In this study, we have examined the early signaling events triggered by a nonmitogenic anti-CD3 mAb. Like the mitogenic anti-CD3 mAb, nonmitogenic anti-CD3 triggered changes in the T cell receptor (TCR) complex, including  $\zeta$  chain tyrosine phosphorylation and ZAP-70 association. However, unlike the mitogenic anti-CD3 stimulation, nonmitogenic anti-CD3 was ineffective at inducing the highly phosphorylated form of  $\zeta$  (p23) and tyrosine phosphorylation of the associated ZAP-70 tyrosine kinase. This proximal signaling deficiency correlated with minimal phospholipase C $\gamma$ -1 phosphorylation and failure to mobilize detectable Ca<sup>2+</sup>. Not only did biochemical signals delivered by nonmitogenic anti-CD3 resemble altered peptide ligand signaling, but exposure of Th1 clones to nonmitogenic anti-CD3 also resulted in functional anergy. Finally, a bispecific anti-CD3  $\times$  anti-CD4 F(ab)<sub>2</sub> reconstituted early signal transduction events and induced proliferation, suggesting that defective association of lck with the TCR complex may underlie the observed signaling differences between the mitogenic and nonmitogenic anti-CD3.

OKT3, a murine antibody specific for the CD3 $\epsilon$  chain of the TCR complex, has been used clinically for over a decade in the treatment of steroid-resistant graft rejection (1). However, use of this antibody has been hampered by a toxic first dose reaction syndrome shown to be related to the initial T cell activation events and ensuing release of cytokines before the suppression of T cell responses (2, 3). Others have demonstrated that the mitogenic activity of OKT3 and other anti-CD3 mAbs depends upon extensive TCR-CD3 cross-linking via binding to FcR-positive cells (4). Therefore, recent efforts have been devoted to developing nonmitogenic forms of anti-CD3 by altering binding to Fc receptor. As a model system, an anti-murine CD3 mAb, 145-2C11, was genetically altered to eliminate FcR binding: its variable region gene was fused to a murine IgG3 Fc region, a mouse isotype with low affinity for murine FcR. This chimeric anti-CD3-IgG3 antibody has been shown to be nonmitogenic in vitro, and did not result in the serum cytokine elevation observed with the whole 145-2C11 mAb in vivo (5). However, the administration of nonmitogenic anti-CD3 mAbs was equally effective in prolonging graft survival as the parental 145-2C11 anti-

body (5). As similar non-FcR-binding mAbs derived from OKT3 are being tested clinically, it is important to gain further understanding of the mechanism(s) by which these nonmitogenic mAbs suppress T cell responses.

The mechanism of immunosuppression by anti-CD3 mAbs is complex. Mitogenic anti-CD3 mAbs modulate the TCR, induce apoptosis, and induce generalized long-term T cell unresponsiveness (6). Similarly, treatment of mice with the nonmitogenic anti-CD3 results in internalization of the TCR complex and depletion of T cells from the circulation and peripheral lymphoid organs. However, in contrast with the mitogenic antibodies, anti-CD3-IgG3 does not appear to induce global T cell unresponsiveness (5). Thus, the different anti-CD3 mAbs may suppress T cell responses by distinct mechanisms. Treatment with anti-CD3-IgG3 alters expression of several T cell surface molecules; both CD44 and Ly-6C are upregulated after exposure to the chimeric anti-CD3 (7). Thus, the interaction of anti-CD3-IgG3 with T cells is not inert, but may deliver at least a partial TCR signal that contributes to its immunosuppressive activity.

Currently, it is thought that TCR signaling results from

a cascade of events requiring the recruitment and activation of nonreceptor tyrosine kinases. One of the earliest consequences of TCR engagement by mAb or peptide-MHC is the tyrosine phosphorylation of components of the TCR complex (8). The  $\zeta$  chain of the TCR complex contains three immunoreceptor tyrosine-based activation motifs (D/EXXYXXL(X)<sub>6-8</sub>YXXL) that become variably phosphorylated after TCR-CD3 ligation (9). It is thought that the activation-induced 21- and 23-kD phosphorylated bands evident on one-dimensional SDS-PAGE represent differentially phosphorylated forms of  $\zeta$  (10). The other CD3 chains,  $\gamma$ ,  $\delta$ , and  $\epsilon$  (containing one immunoreceptor tyrosine-based activation motif each), become tyrosine phosphorylated as well (8). It has been hypothesized that the src family kinases, lck or fyn, may be responsible for these early phosphorylation events (9). Within minutes, additional tyrosine phosphorylated proteins, including the ZAP-70 kinase, associate with the TCR-CD3 complex (11). These proximal events lead to a series of biochemical signals that activate downstream substrates in the PI-3 kinase, Ras, and phospholipase C $\gamma$ -1 (PLC $\gamma$ -1)<sup>1</sup> pathways, ultimately leading to activation of the T cell (9).

Until recently, it was thought that this cascade of events was always fully engaged after exposure to peptide-MHC ligand or mAbs and that different responses to stimuli reflected a quantitative addition of the number of receptors engaged. However, antigenic peptide analogues, designated as altered peptide ligands (APL), have illustrated that the TCR is not an on-off switch. Rather, stimulation with APL can result in qualitative differences in the early signals transduced through the TCR. Specifically, stimulation with APL results in a characteristic biochemical pattern involving partial  $\zeta$  phosphorylation and ZAP-70 association in the absence of phosphorylation, ultimately leading to a lack of inositol-trisphosphate (IP<sub>3</sub>) turnover (10, 12, 13). The delivery of such a partial signal effectively shuts down T cell clones, resulting in the induction of unresponsiveness as manifested by an inability of the anergized T cell clones to produce IL-2 when rechallenged under optimal conditions.

In the present study, we examined the effect of nonmitogenic anti-CD3 mAbs on early signaling events in T cell clones. The anti-CD3-IgG3 chimeric antibody transduced a signal characterized by an altered pattern of  $\zeta$  chain and ZAP-70 tyrosine phosphorylation, leading to defects in downstream signaling events. Nonmitogenic anti-CD3 also failed to induce higher order aggregation of the TCR into a cap. Similar signaling changes were observed in naive T cells at the level of TCR complex and PLC $\gamma$ -1 tyrosine phosphorylation. Specific defects in early signal transduction and mitogenicity were restored by coaggregating CD4 (and thus presumably lck) with the TCR-CD3 complex. The nonmitogenic anti-CD3 induced T cell unresponsiveness in Th1 clones but not naive T cells. Together, these results suggest that administration of nonmitogenic anti-CD3 mAbs,

especially in recipients undergoing acute transplant rejection, may result in the delivery of a partial T cell signal that renders activated T cells unresponsive.

## Materials and Methods

**Animals.** 6- to 8-wk-old BALB/c, DBA/2J, and B10.A mice were purchased from Frederick Cancer Research Institute Laboratories (Frederick, MD). DO 11.10 mice, transgenic for an OVA peptide (323-339)-specific, I-A<sup>d</sup>-restricted  $\alpha\beta$ -TCR, were a gift of Drs. D. Loh and K. Murphy (Washington University, St. Louis, MO) (14). All mice were bred and maintained in a specific pathogen-free facility at the University of Chicago.

**Cell Lines, Antibodies, and Reagents.** The chicken OVA-specific Th1 clone pGL10 was provided by Dr. F. Fitch (University of Chicago, Chicago, IL) and the pigeon cytochrome c (PCC)-specific Th1 clone, AE.7, was provided by Dr. M. Jenkins (University of Minnesota, Minneapolis, MN). OVA and PCC were purchased from Sigma Chem. Co. (St. Louis, MO). The following mAbs were used in this study: 145-2C11 (anti-CD3), AT83A (anti-Thy-1) (provided by Dr. F. Fitch, University of Chicago, Chicago, IL); anti-CD3-IgG3 (5, 7); the anti-Ig antisera: goat anti-mouse IgG3 (Sigma), rabbit anti-mouse IgG3 (Zymed, San Francisco, CA), rabbit anti-hamster (Cappel, Durham, NC); 145-2C11-FITC (Boehringer Mannheim, Indianapolis, Indiana); PV-1 (anti-CD28) (Dr. C. June, Naval Medical Research Institute, MD); H146 (anti- $\zeta$  mAb containing supernatant) (Dr. F. Fitch); 4G10 (anti-phosphotyrosine) and anti-PLC $\gamma$ -1 (mixed mAbs) (UBI, Lake Placid, NY); 12-222 (anti-ZAP-70 antiserum) (Dr. A. Weiss, University of California, San Francisco, CA). Cyclosporine A was purchased from Sandoz (Basel, Switzerland). Indo-1 was purchased from Molecular Probes (Eugene, OR).

**Anti-CD3-Fos  $\times$  Anti-CD4-Jun Bispecific F(ab'-Zipper)<sub>2</sub> Production.** The anti-CD3 antibody was derived from hamster anti-mouse CD3 hybridoma 145-2C11 (15), and the anti-CD4 antibody from rat anti-mouse CD4 hybridoma GK1.5 (16). The VH and VL sequences for 145-2C11 (5), (GenBank accession no. U17870 and U17871) and GK1.5 (16, 16a) had been determined. Homodimers of anti-CD3-Fos and anti-CD4-Jun F(ab'-zipper)<sub>2</sub> were expressed by the genetic method described by Kostelny et al., (17). Anti-CD3-Fos and anti-CD4-Jun were individually purified from transfected Sp2/0 spent media by protein G-Sepharose affinity chromatography (18). The two homodimers were then reduced and reoxidized to form bispecific F(ab'-zipper)<sub>2</sub> as described (17). Bispecific F(ab'-zipper)<sub>2</sub> was further purified by BAKERBOND Abx column chromatography or hydrophobic interaction chromatography on a Bio-Gel® Phenyl-5 PW column.

**Proliferation Assays.** pGL10 and AE.7 T cell clones were maintained as previously described (19, 20). T cell clones were purified by Ficoll-Hypaque density centrifugation before use in all experiments. BALB/c spleens were lysed in hypotonic ACK buffer to remove erythrocytes. Proliferation and unresponsiveness assays were in 5 or 10% FCS supplemented DMEM. In a 96-well flat-bottomed plate,  $2 \times 10^5$  splenocytes or  $1 \times 10^5$  pGL10 T cells were incubated on ice for 10 min with anti-CD3 (final concentration of 1  $\mu$ g/ml), and then for another 10 min on ice with the appropriate cross-linker (rabbit anti-IgG3 at 1:30 or goat anti-IgG3 at 1:100 or goat anti-hamster at 1:300), before being placed at 37°C. For anti-CD3-Fos homodimer or anti-CD3  $\times$  anti-CD4 heterodimer proliferation assays, antibodies were serially diluted in 96-well flat-bottomed plates, starting at 10  $\mu$ g/ml. Assays were pulsed with 1  $\mu$ Ci/well of [<sup>3</sup>H]thymidine for the last 8 h of a 48-h incubation, harvested on a Filtermate 196 96-well plate harvester

<sup>1</sup>Abbreviations used in this paper: APL, altered peptide ligands; IP<sub>3</sub>, inositol-trisphosphate; PCC, pigeon cytochrome C; PLC $\gamma$ -1, phospholipase C $\gamma$ -1.

(Packard Instrument Co., Meriden, CT), and counted on a Packard TopCount microplate scintillation counter. Results are presented as the mean of triplicate cultures. Standard errors were less than 20% of the mean.

For induction of unresponsiveness, 24-well plates were preblocked with 10% FCS-supplemented DMEM overnight to prevent soluble anti-CD3-IgG3 from adhering to the plastic. DO 11.10 lymph node cells ( $2 \times 10^6$ ) or pGL10 clones ( $1 \times 10^6$ ) were incubated 24 h in 1 ml media with or without 1–10  $\mu\text{g/ml}$  soluble anti-CD3-IgG3, CsA (1  $\mu\text{g/ml}$ ), anti-CD28 (1  $\mu\text{g/ml}$ ),  $2\text{--}3 \times 10^6$  T-depleted irradiated BALB/c splenocytes. Cells were then washed three times, and rested 72 h at  $37^\circ\text{C}$ . TCR reexpression was verified via FACS<sup>®</sup> analysis. For the secondary stimulation,  $4\text{--}5 \times 10^4$  DO 11.10 lymph node cells or pGL10 cells were plated in the presence of  $2\text{--}5 \times 10^5$  T-depleted (anti-Thy-1 plus complement) irradiated splenocytes and 1  $\mu\text{g/ml}$  soluble 145-2C11 or 800  $\mu\text{g/ml}$  OVA. Cultures were pulsed with [ $^3\text{H}$ ]thymidine after 48 h. For IL-2 production,  $2.5 \times 10^4$  cells per well were stimulated in a 96-well flat-bottomed plate with immobilized anti-CD3 plus anti-CD28 at 1  $\mu\text{g/ml}$ . 24 h supernatants from 3 wells were pooled and analyzed by ELISA (Endogen, Cambridge, MA). For AE.7 assays,  $1 \times 10^6$  T cells per well were incubated for 24 h with 1  $\mu\text{g/ml}$  of anti-CD3-IgG3 mAb, washed, rested, and then in a 96-well flat bottomed plate,  $4 \times 10^4$  T cells were restimulated in the presence of  $5 \times 10^5$  T-depleted irradiated B10.A splenocytes and 10  $\mu\text{M}$  PCC.

**Biochemistry.** T cell clones or BALB/c lymph node cells were washed in PBS and then resuspended in ice-cold PBS at  $1 \times 10^8/\text{ml}$  or  $2 \times 10^8/\text{ml}$ , respectively. Anti-CD3-IgG3 was added at 4–5  $\mu\text{g/ml}$  for a 10-min incubation on ice. An equal volume of anti-Ig cross-linker or PBS prewarmed to  $37^\circ\text{C}$  was added and samples were incubated a further 2.5–5 min in a  $37^\circ\text{C}$  water bath. For anti-CD3-Fos homodimer and anti-CD3  $\times$  anti-CD4 heterodimer stimulations, cells were stimulated with 10  $\mu\text{g/ml}$  of antibody. After the incubation, an equal volume of ice-cold  $2\times$  lysis buffer was added (final concentration: 0.5% Triton X-100, 50 mM Tris [pH 7.6], 100 mM NaCl, 5 mM EDTA, 1 mM  $\text{Na}_3\text{VO}_4$ , 10  $\mu\text{g/ml}$  each leupeptin and aprotinin, 25  $\mu\text{M}$  NPGF, and 1 mM PMSF). For immunoprecipitations, 20  $\lambda$  of a 50% protein A-agarose bead slurry (Pharmacia-Upjohn, Uppsala, Sweden) were coated with 200  $\lambda$  of mAb-containing supernatant or 2  $\lambda$  of antisera for 1 h at  $4^\circ\text{C}$ . Lysates were added to the precoated protein A-agarose beads and incubated 1 h at  $4^\circ\text{C}$ . The samples were resolved on a 12% SDS-polyacrylamide gel for  $\zeta$  immunoprecipitations or an 8% gel for PLC $\gamma$ -1, and then transferred to PVDF membrane (Millipore, Bedford, MA). Blots were blocked with 10% BSA (Sigma, St. Louis, MO). After incubation with primary antibody and the appropriate horseradish-peroxidase-coupled secondary antibody, blots were developed by Enhanced Chemilumi-

nescence (Amersham Corp., Arlington Heights, IL). Densitometry measurements were performed using an AMBIS Image Acquisition and Analysis instrument (San Diego, CA).

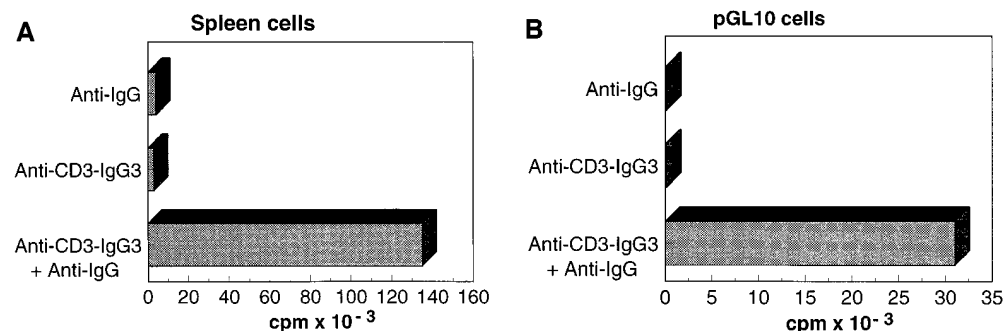
**Calcium Flux.** pGL10 were washed with DMEM containing 10 mM Hepes at pH 7.0 and then incubated at  $5 \times 10^6$  cells/ml with 5  $\mu\text{M}$  indo-1 at  $37^\circ\text{C}$  for 30 min. An equal volume of DMEM with Hepes and 5% FCS at pH 7.4 was added and cells were incubated 30 min. Cells were washed twice with 5% FCS-supplemented DMEM at pH 7.2 and resuspended at  $1 \times 10^6/\text{ml}$  for data acquisition on a FACStar<sup>®</sup> plus (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Cells were briefly warmed before stimulation with anti-CD3-IgG3 (1  $\mu\text{g/ml}$ ) plus rabbit anti-IgG3 (1:30). An increase in the 404:495 nm indo-1 emission ratio is indicative of a rise in intracellular  $\text{Ca}^{2+}$ . Results were analyzed using Multitime (Phoenix Flow Systems, San Diego, CA).

**Confocal Microscopy.** Purified DO 11.10 lymph node T cells or pGL10 cells ( $10^6$ ) were incubated with 5  $\mu\text{g/ml}$  145-2C11-FITC on ice for 10 min and then stimulated with an equal volume of  $37^\circ\text{C}$  prewarmed goat anti-hamster in PBS (1:300 final) for 0 or 5 min at  $37^\circ\text{C}$ . T cells were transferred to a polystyrene FACS<sup>®</sup> tube and fixed in 3% paraformaldehyde for 10 min at room temperature, washed three times with PBS, and then resuspended in 25  $\lambda$  of mounting solution (0.5 mg/ml O-Phenylenediamine, 90% glycerol, 0.05 M Tris [pH 8.0], 0.2%  $\text{NaN}_3$ ). Samples were analyzed on a ZEISS 410 confocal microscope.

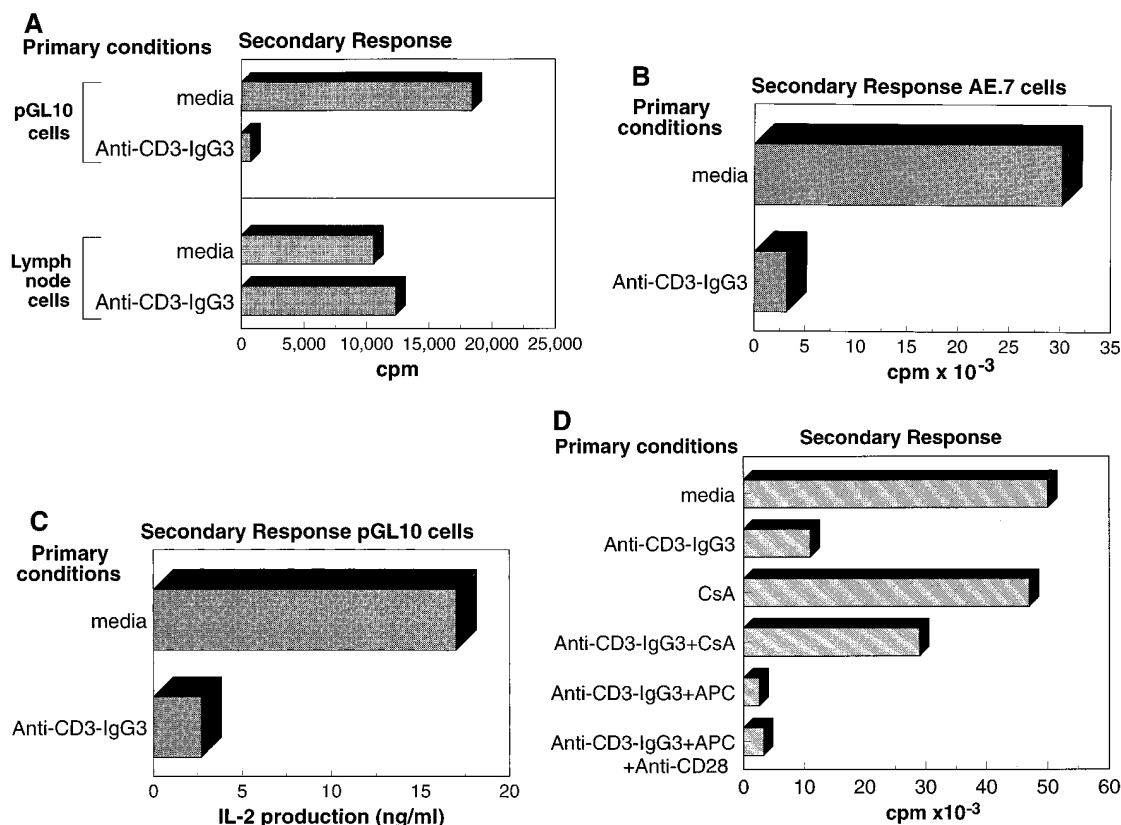
## Results

*The anti-CD3-IgG3 mAb Is Nonmitogenic Due to Insufficient Cross-linking of the TCR.* Unlike the original 145-2C11 mAb, the anti-CD3-IgG3 chimeric antibody does not induce proliferation or IL-2 production in whole spleen cells (5). Also, soluble 145-2C11 failed to induce proliferation of T cell clones in the absence of FcR-mediated cross-linking (Bluestone, J.A. and J.A. Smith, unpublished observations; data not shown). To test directly the role of multivalent cross-linking, a secondary IgG3-specific cross-linking antibody was added to cultures containing the anti-CD3-IgG3 mAb. The addition of the cross-linking reagent reconstituted a mitogenic stimulus for both fresh murine splenocytes and a T cell clone (Fig. 1). Thus, the induction of proliferation by anti-CD3 requires a higher order of TCR aggregation that cannot be achieved by bivalent Ab binding alone.

*Nonmitogenic Anti-CD3 Renders T Cell Clones Hyporesponsive.* Although insufficient for induction of T cell proliferation or cytokine production, the anti-CD3-IgG3



**Figure 1.** Non-FcR-binding anti-CD3 induces proliferation only in the presence of cross-linking anti-Ig antibody. Whole spleen (top) or pGL10 clone cells (bottom) were cultured with an anti-CD3-IgG3 chimeric antibody and a secondary rabbit anti-mouse IgG3 Ab mAb for 48 h. Results are expressed as the mean of triplicate determinations and are representative of four independent experiments.

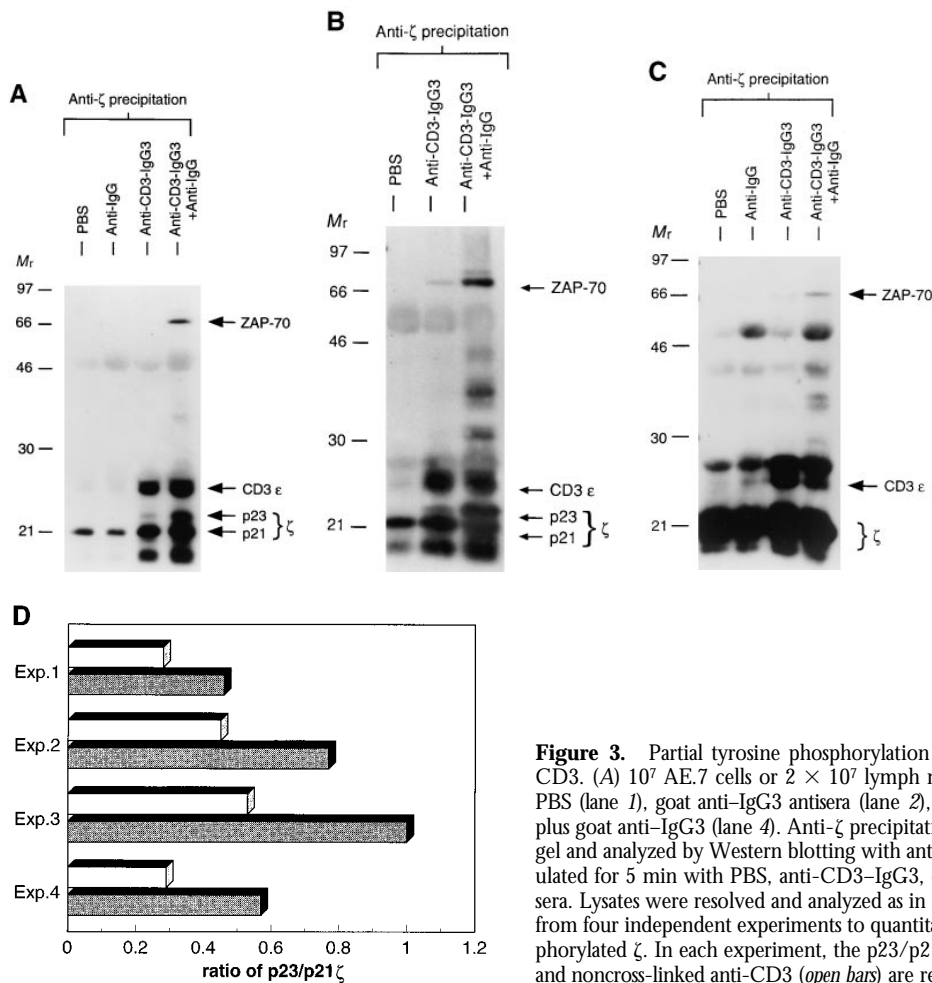


**Figure 2.** T cell clones, but not lymph node T cells, are hyporesponsive after exposure to nonmitogenic anti-CD3. (A) DO 11.10 lymph node cells or pGL10 cells were incubated with either media alone or anti-CD3-IgG3 in the presence of irradiated T-depleted spleen cells for 24 h, washed, and rested for 72 h. The T cells were restimulated with mitogenic anti-CD3 (145-2C11) and fresh APC. (B) AE.7 clone cells were incubated with or without nonmitogenic anti-CD3, washed, and rested as above, and restimulated with the antigen PCC plus fresh APC. (C) pGL10 cells were incubated with or without nonmitogenic anti-CD3. After the 72-h rest, the pGL10 were restimulated with immobilized anti-CD3 plus anti-CD28. Culture supernatants were analyzed by IL-2 ELISA. (D) pGL10 cells were incubated with nonmitogenic anti-CD3 in the presence of cyclosporine A, splenic APC, and anti-CD28 as indicated. 72 h after the primary culture, cells were restimulated with OVA antigen and APC. A, C, and D are representative of two separate experiments, and B is representative of four experiments.

mAb may deliver at least a partial signal, which alters T cell function. Therefore, the effects of anti-CD3-IgG3 on the functional responses of naive cells and Th1 clones were examined. pGL10 T cells or DO11.10 lymph node cells were cultured in the presence of splenic accessory cells (to compensate for the presence of non-T cells in the naive population) and nonmitogenic anti-CD3-IgG3. Previous studies have shown that treatment of T cells with anti-CD3-IgG3 resulted in downmodulation of TCR expression within 24 h (7). Therefore, after 24 h, the cells were washed, and recultured for 3 d to allow reexpression of the TCR (data not shown). As seen in Fig. 2 A, upon restimulation with the mitogenic 145-2C11 mAb plus splenic APCs, thymidine incorporation by anti-CD3-IgG3-treated pGL10 was markedly reduced as compared with pGL10 cultured with media alone. In contrast, the functional responses of murine lymph node T cells were not affected by culture with anti-CD3-IgG3. The clonal unresponsiveness did not merely reflect decreased viability, because anti-CD3-IgG3-treated clones proliferated in the presence of exogenously added IL-2 (data not shown). The effect of anti-CD3-IgG3 was not specific

to the pGL10 clone, because the nonmitogenic anti-CD3 rendered the PCC-specific clone, AE.7, hyporesponsive as well (Fig. 2 B). To determine whether the reduced proliferation of anti-CD3-IgG3-treated T cell clones correlated with IL-2 production, pGL10 clones were cultured with or without anti-CD3-IgG3 for 24 h, rested, and then restimulated with immobilized anti-CD3 plus anti-CD28 (PV-1), conditions known to induce readily detectable IL-2 production (Fig. 2 C). Anti-CD3-IgG3-treated clones secreted significantly less IL-2 than the media-treated control cells. These data indicated that exposure to soluble, non-cross-linked anti-CD3 selectively reduces the responsiveness of Th1 clones as compared with naive cells.

To examine whether the presence of CsA or CD28 costimulation would affect the nonmitogenic anti-CD3-induced unresponsiveness, pGL10 T cells were cultured with nonmitogenic anti-CD3 alone, or nonmitogenic anti-CD3 in the presence of CsA, or splenic APCs and anti-CD28 (Fig. 2 D). CsA partially blocked the induction of unresponsiveness by nonmitogenic anti-CD3, suggesting that this process may depend upon a calcium signal. In contrast, addition



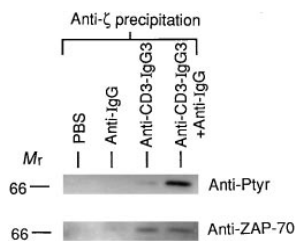
**Figure 3.** Partial tyrosine phosphorylation of TCR components by nonmitogenic anti-CD3. (A)  $10^7$  AE.7 cells or  $2 \times 10^7$  lymph node cells (C) were stimulated for 2.5 min with PBS (lane 1), goat anti-IgG3 antisera (lane 2), anti-CD3-IgG3 (lane 3), or anti-CD3-IgG3 plus goat anti-IgG3 (lane 4). Anti- $\zeta$  precipitations were resolved by electrophoresis on a 12% gel and analyzed by Western blotting with anti-phosphotyrosine. (B) pGL10 cells were stimulated for 5 min with PBS, anti-CD3-IgG3, or anti-CD3-IgG3 plus rabbit anti-IgG3 antisera. Lysates were resolved and analyzed as in A. (D) Densitometry was performed on results from four independent experiments to quantitate the relative amounts of p21- and p23-phosphorylated  $\zeta$ . In each experiment, the p23/p21 ratios for cross-linked anti-CD3 (hatched bars) and noncross-linked anti-CD3 (open bars) are represented.

of anti-CD28 mAb in the primary culture failed to restore secondary responses.

**Nonmitogenic Anti-CD3 Delivers a Partial TCR Signal.** The functional consequences of culture with nonmitogenic anti-CD3 support the hypothesis that anti-CD3-IgG3 delivers a signal. Therefore, experiments were performed to determine the nature of the TCR signal triggered by nonmitogenic anti-CD3. Upon ligation of the TCR, one of the earliest events to occur is the tyrosine phosphorylation of components of the TCR complex ( $\zeta$  and CD3 $\epsilon$ , CD3 $\delta$ , and CD3 $\gamma$ ) (8). Phosphorylation of these chains allows subsequent association and phosphorylation of a variety of other proteins, including the protein tyrosine kinase, ZAP-70 (9). In the present study, the ability of nonmitogenic anti-CD3 to trigger these crucial proximal events in T cell clones was examined. T cells were stimulated with the anti-CD3-IgG3 mAb in the presence or absence of a secondary Ig cross-linker. The TCR complex was immunoprecipitated with anti- $\zeta$  and analyzed for tyrosine phosphorylation (Fig. 3 A). Stimulating T cells with anti-CD3 under cross-linking conditions induced both 21- and 23-kD forms of phosphorylated  $\zeta$  (p21 and p23) as well as phosphorylation of CD3 $\epsilon$ . The phosphorylated band below p21 (~18 kD) most likely represents another isoform of phosphory-

lated  $\zeta$  (21). In contrast, the noncross-linked anti-CD3-IgG3 mAb induced similar levels of phosphorylated CD3 $\epsilon$  and p21  $\zeta$ , but significantly less p23  $\zeta$ . Quantitation of the p21 and p23 bands by densitometry in multiple T cell clone experiments ( $n = 4$ ) revealed a consistent correlation between the degree of anti-CD3 cross-linking and the p23/p21 ratio; conditions that promote cross-linking increased the relative level of p23 expression (Fig. 3 D).

Examination of the phosphoproteins that coprecipitated with the  $\zeta$  chain in darker exposures or greater cell number revealed further differences between anti-Ig cross-linked and noncross-linked conditions (Fig. 3 B). Unlike the cross-linked anti-CD3 stimulation, several of these phosphoproteins (bands between 30 and 46 kD as well as at 70 and 76 kD) were missing or reduced in the anti- $\zeta$  precipitations from T cells stimulated with the noncross-linked anti-CD3 mAb. The proximal signals triggered by nonmitogenic anti-CD3 in lymph node T cells were similar to those induced in clones in that (a) nonmitogenic anti-CD3 induces phosphorylation of TCR chains and (b) in the absence of cross-linking, several TCR-associated phosphotyrosine containing proteins are missing or reduced in intensity (Fig. 3 C). Thus, in both T cell clones and naive T cells, the anti-CD3-IgG3-induced TCR activation complex (with asso-



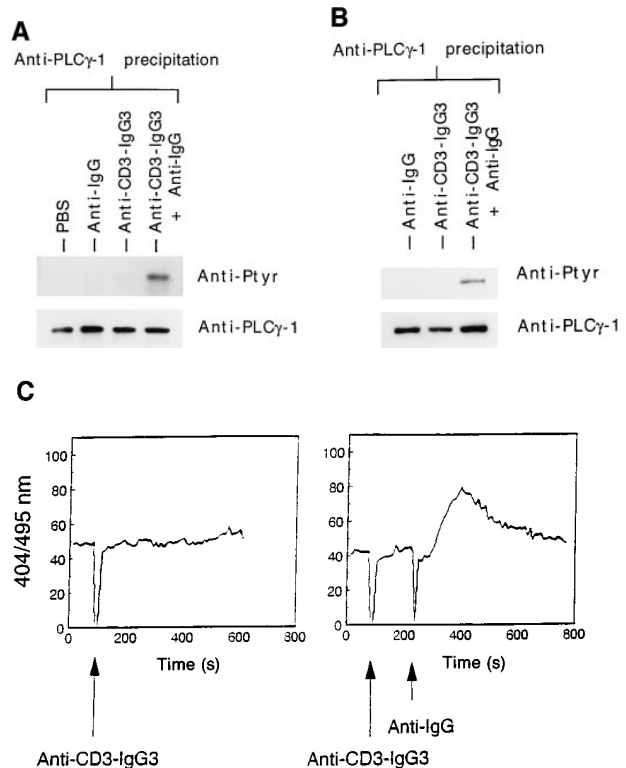
**Figure 4.** Stimulation with non-cross-linked anti-CD3 is sufficient for TCR association, but not phosphorylation of ZAP-70.  $2 \times 10^7$  pGL10 T cells were stimulated with PBS, goat anti-IgG3 alone, anti-CD3-IgG3, or anti-CD3-IgG3 plus anti-IgG, for 2.5 min at 37°C, lysed, and immunoprecipitated with anti- $\zeta$ . Blots were probed with anti-ZAP-70 (bottom), and then stripped and reprobed with anti-phosphotyrosine (top).

ciated signaling molecules) contained fewer tyrosine phosphorylated molecules than a mitogenically stimulated TCR complex. These results suggest that although nonmitogenic anti-CD3 induces some tyrosine phosphorylation of  $\zeta$  and the CD3 chains, it is deficient in triggering other proximal signaling events.

Previous studies have shown that the 70-kD band observed in anti- $\zeta$  precipitates represents the TCR-associated tyrosine kinase, ZAP-70 (22). The reduced intensity of this band in the immunoprecipitates from nonmitogenic anti-CD3-treated cells could either represent a failure of ZAP-70 association or deficient phosphorylation. To address this issue, T cells were stimulated with cross-linked or non-cross-linked anti-CD3, and the lysates were immunoprecipitated with anti- $\zeta$ . The Western blot was probed with an anti-ZAP-70 antisera. As seen in Fig. 4, the noncross-linked anti-CD3-IgG3 induced similar levels of ZAP-70 recruitment to the TCR complex; yet as confirmed by re-probing the blot with anti-phosphotyrosine, the proportion of ZAP-70 that was tyrosine phosphorylated was significantly reduced. Thus, in the absence of CD3 cross-linking, ZAP-70 associates with the TCR-CD3 complex, but it is not efficiently phosphorylated.

**Defects in Downstream Events in the Absence of TCR-CD3 Cross-linking.** The differences in proximal signal transduction observed in the absence of cross-linking were likely to be reflected in critical downstream biochemical events, such as the tyrosine phosphorylation (and thus activation of) PLC $\gamma$ -1. To examine this event, pGL10 were stimulated with anti-CD3-IgG3 in the presence or absence of cross-linker (Fig. 5 A). The dramatic increase in PLC $\gamma$ -1 phosphorylation observed in the presence of a secondary cross-linking Ab was not observed following anti-CD3-IgG3 stimulation alone. Similarly, cross-linking with anti-IgG enhanced PLC $\gamma$ -1 tyrosine phosphorylation induced by the nonmitogenic anti-CD3 in naive cells (Fig. 5 B).

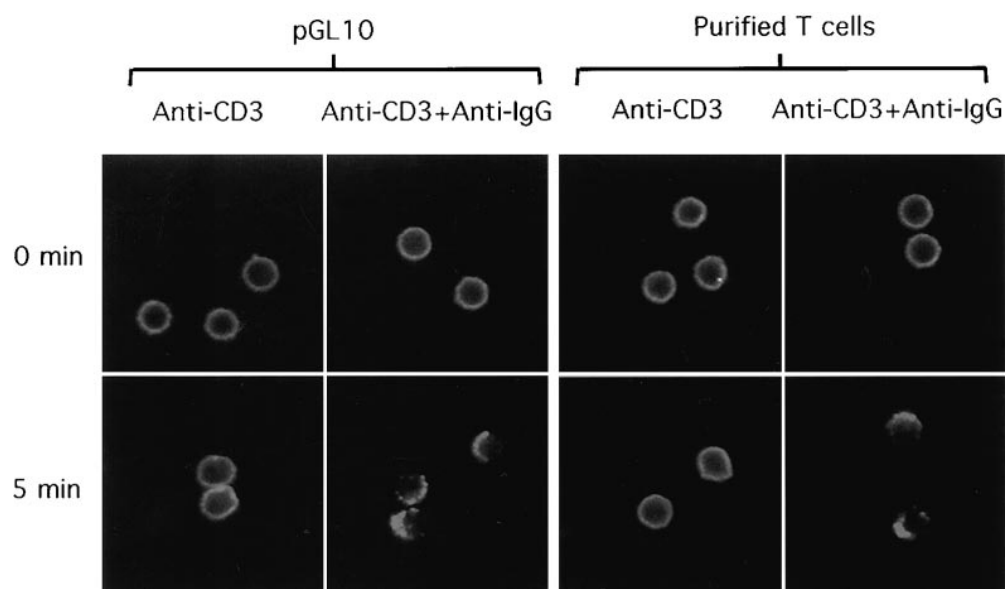
Because anti-CD3-IgG3 was unable to induce significant PLC $\gamma$ -1 phosphorylation, it was anticipated that one of the events that depends upon PLC $\gamma$ -1 activation,  $\text{Ca}^{2+}$  mobilization, would likewise be impaired. T cell clones were loaded with the calcium-sensitive dye indo-1 and then analyzed by FACS<sup>®</sup> for calcium flux. A calcium flux was not detected when the cells were stimulated with the anti-CD3-IgG3 alone, even after 5 min. However, in T cells incubated with anti-CD3-IgG3 followed by the addition of a secondary cross-linker, a characteristic calcium flux was



**Figure 5.** Impaired PLC $\gamma$ -1 activation and  $\text{Ca}^{2+}$  flux in the absence of anti-CD3 cross-linking. (A)  $2 \times 10^7$  pGL10 cells were stimulated for 5 min at 37°C as indicated. Samples were precipitated with anti-PLC $\gamma$ -1, and then resolved on an 8% gel. The Western blot was probed with anti-phosphotyrosine (top), stripped, and then reprobed with anti-PLC $\gamma$ -1 (bottom). (B)  $4 \times 10^7$  lymph node cells were stimulated for 5 min with PBS, anti-CD3-IgG3, or anti-CD3-IgG3 plus goat anti-IgG3 and analyzed as in A. Densitometry performed on this experiment showed a fourfold increase in PLC $\gamma$ -1 phosphorylation (with compensation for protein amount) upon anti-CD3 cross-linking. (C) T cell clones were loaded with the calcium-sensitive dye indo-1, stimulated with anti-CD3-IgG3 (left), or anti-CD3-IgG3 followed by rabbit anti-IgG3 (right). Cells were analyzed on a FACStar Plus<sup>®</sup> for calcium flux. The rise in relative intracellular calcium concentration is indicated by an increase in the 405/495 nm emission ratio. (A–C) Data is representative of two separate experiments.

observed within 1 min (Fig. 5 C). Anti-IgG Abs in the absence of anti-CD3 did not result in a calcium flux (data not shown). These results demonstrate that the downstream signaling events of PLC $\gamma$ -1 activation and the ensuing  $\text{Ca}^{2+}$  flux are dependent upon extensive cross-linking of the TCR-CD3 by anti-CD3 mAbs.

Aggregation of the TCR complex has been shown to correlate with T cell activation; Kupfer et al. (23) demonstrated that when T cells encounter antigen-MHC on APCs, the TCR redistributes on the cell surface to form an aggregated activation cap. This redistribution is a signaling-dependent process most likely involving reorganization of the cytoskeleton (24, 25). To test whether the addition of a cross-linking Ab to anti-CD3 results in an aggregated TCR cap, confocal microscopy was performed on pGL10 and purified DO11.10 T cells incubated with anti-CD3 (2C11-FITC) under cross-linking versus noncross-linking conditions (Fig. 6). In the presence of cross-linking Ab, anti-



**Figure 6.** In the presence of a secondary cross-linker, anti-CD3 induces formation of an aggregated TCR cap. pGL10 T cells (left two columns) or purified DO 11.10 T cells (right two columns) were stimulated with anti-CD3-FITC at 37°C in the absence (left for each T cell type) or presence (right for each cell type) of goat anti-hamster cross-linking anti-sera. Cells were fixed at 0 min (top row) and 5 min (bottom row) after addition of cross-linker, and analyzed by confocal microscopy.

CD3 stimulation induces aggregation of the TCR into a cap on one side of the cell. However, in the absence of cross-linker, the anti-CD3 remained diffusely distributed on the cell surface. Thus, the signal delivered by nonmitogenic anti-CD3 appeared insufficient for the redistribution of TCRs into an aggregated cap.

*Recruitment of CD4-lck into the Complex Reconstitutes Complete Proximal Signal Transduction and Mitogenicity.* The inability of nonmitogenic anti-CD3 to trigger specific downstream events and proliferation most likely stems from the defective proximal events observed involving  $\zeta$  and ZAP-70. Previous studies have suggested that the src family kinase, lck, plays a crucial role in the phosphorylation of  $\zeta$ , which subsequently allows association and phosphorylation of ZAP-70 (26, 27). Thus, it was possible that the differences in  $\zeta$  and ZAP-70 phosphorylation seen upon the addition of cross-linker to anti-CD3 may have reflected increased lck activation or enhanced recruitment to the TCR. Initial experiments examining lck activation by monitoring lck tyrosine phosphorylation revealed no differences between cross-linking and noncross-linking conditions. It is clear that CD4 associates with lck and can interact with the TCR complex inducibly upon TCR ligation of antigen-MHC. Thus, artificially bringing CD4-lck into the TCR complex might reconstitute a mitogenic anti-CD3 stimulus, even in the absence of a secondary cross-linking Ab. To test this hypothesis, we took advantage of a bispecific anti-CD3  $\times$  anti-CD4 reagent prepared by a molecular approach to insure the presence of monovalent arms specific for CD3 and CD4 (as described in Materials and Methods). T cells were incubated with anti-CD3-Fos homodimer or the anti-CD3  $\times$  anti-CD4 bispecific F(ab) $'_2$ , lysed, and the TCR-CD3 complex was then immunoprecipitated and analyzed. The bispecific construct induced significant p23  $\zeta$ , ZAP-70 phosphorylation, as well as association of the phosphoproteins between 30–46 kD (Fig. 7 A), even in the absence of a secondary cross-linking antibody. In contrast,

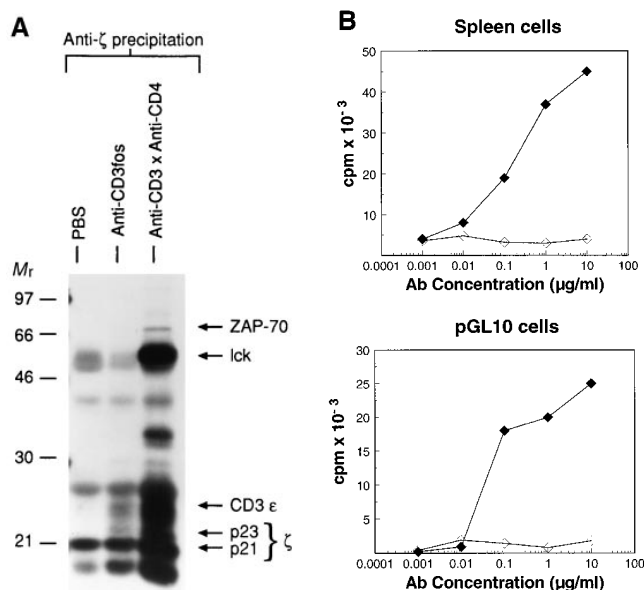
the overall pattern induced by anti-CD3-Fos resembled the results seen in T cells stimulated with the anti-CD3-IgG3 mAb: specifically, a reduced association of phosphoproteins and barely detectable ZAP-70 phosphorylation. In the lysates of T cells stimulated with the bispecific anti-CD3  $\times$  anti-CD4 construct, a large tyrosine-phosphorylated protein was observed that migrated just above the heavy chain. This phosphoprotein is likely to be p56 lck based on protein size. This band never appeared in the cross-linked anti-CD3 studies. One possible explanation for this difference is that in the absence of CD4 coaggregation, lck may dissociate from the TCR complex after lck phosphorylates its substrates. Whereas under stimulation conditions using the bispecific antibody, lck remains in the complex longer due to stable association with coaggregated CD4.

The biochemical results suggested that the anti-CD3  $\times$  anti-CD4 bispecific antibody delivered a competent activating signal to the T cells. In fact, T cell clones or fresh murine T cells cultured in the presence of anti-CD3  $\times$  anti-CD4 heterodimer proliferated, whereas T cells cultured with the anti-CD3-Fos homodimer did not (Fig. 7 B). Thus, enhanced association of lck with the TCR complex reconstituted both early signaling events and a mitogenic stimulus in the absence of further Ab cross-linking.

## Discussion

In this study, we have shown that bivalent anti-CD3 delivers a partial TCR signal that renders Th1 clones hyporesponsive. This signal consists of phosphorylation of several components of the TCR complex, (bands representing CD3 $\epsilon$ , CD3 $\delta$ ), ZAP-70 association, and partial phosphorylation of TCR  $\zeta$ ; in the absence of cross-linking, there is a relatively greater induction of the phosphorylated p21  $\zeta$  as compared with the p23  $\zeta$  band species evident in T cell clones. Presently, it is unclear what the p21 and p23 forms of  $\zeta$  represent. p21 induction appears to be sufficient for





**Figure 7.** Stimulation with anti-CD3  $\times$  anti-CD4 heterodimer results in increased phosphorylation of proteins associated with the TCR complex and reconstitutes a mitogenic stimulus. (A)  $10^7$  pGL10 T cells were stimulated with the anti-CD3-Fos F(ab) $'_2$ , or the bispecific F(ab) $'_2$  (anti-CD3  $\times$  anti-CD4) for 5 min at 37°C. Samples were precipitated with anti- $\zeta$ , and blots were probed with anti-phosphotyrosine as in Fig. 2 A. (B) Whole spleen (top) or pGL10 T cells (bottom) were cultured with serial log dilutions of anti-CD3-Fos homodimer (open diamonds) or bispecific anti-CD3  $\times$  anti-CD4 (closed diamonds) for 48 h. Data is representative of two (A) and three (B) separate experiments.

association of the ZAP-70 kinase with the TCR complex, whereas p23 induction and ZAP-70 phosphorylation appear to be interrelated events. Indeed, the low level of ZAP-70 phosphorylation observed in the noncross-linked situation correlates with the small amount of p23  $\zeta$  that is generated. In a recent study, Weist et al. (28) proposed that the p23 form of  $\zeta$  observed in thymocytes upon *in vitro* stimulation depends on greater TCR aggregation. Our findings are consistent with this hypothesis. Higher orders of TCR aggregation also appear to be required for recruitment of other phosphotyrosine containing molecules to the TCR-CD3 complex in both T cell clones and bulk naive cells. If any of these tyrosine-phosphorylated molecules contain SH2 domains, they may require the fully phosphorylated p23 form of  $\zeta$  for association. Alternatively, the p23 form may be required for docking of a kinase that phosphorylates these associated molecules.

The observation that noncross-linked anti-CD3 induces less ZAP-70 phosphorylation and p23 phospho- $\zeta$  bears a striking resemblance to the findings in the altered peptide ligand studies (10, 13). The relative contribution of affinity for MHC or TCR (and thus occupancy) versus TCR aggregation has been unclear in these systems. Recently, Lyons et al. (29) showed a correlation between antagonist activity of certain altered peptide ligands and a higher TCR dissociation rate. However, this finding does not exclude a role for aggregation in that a shorter dwell time of the TCR may fail to induce the oligomerization required for a fully activating stimulus. In the present study, the issue of

affinity has been addressed; the same primary antibody was used in both cross-linked and noncross-linked situations; thus, intrinsic affinity for TCR was held constant. Because similar signaling deficits were found in noncross-linked anti-CD3 and altered peptide ligand stimulations, it is possible that the altered peptide ligands may induce their characteristic partial signals because of insufficient TCR aggregation.

There are several ways in which the localization of multiple TCR complexes within a large aggregate could enhance signaling. In the kinetic proofreading model proposed by McKeithan (30), TCR signal transduction was modeled as a reversible multistep pathway containing sequential phosphorylation events. In this paradigm, aggregation of TCRs might enhance propagation of the signal by favoring phosphorylation over dephosphorylation. On a more mechanistic level, aggregation may aid in recruiting key signaling molecules; recruitment of molecules may be further stabilized if there are multiple potential contact points (catalytic sites, SH2 domains, or other recognition motifs) between components of the TCR complex that are in close proximity. For example, if lck binds one phosphorylated ZAP-70 through its SH2 domain, the lck would be in a prime position to phosphorylate a neighboring ZAP-70 molecule in the TCR aggregate. In the noncross-linked situation, lck might migrate away before phosphorylating more ZAP-70 molecules. Thus, aggregated TCR signal transduction may result in amplification of these signals, because one kinase may act on multiple substrates. This capacity for amplification would mean that proximal differences should become magnified as the signal is propagated. As seen in the present study, a relative reduction in ZAP-70 phosphorylation leads to a more dramatic deficiency in PLC $\gamma$ -1 phosphorylation and Ca $^{2+}$  flux.

The redistribution of TCR complexes to one pole, within minutes upon addition of secondary cross-linker to anti-CD3, is likely to reflect changes in the underlying cytoskeleton. Others have shown that TCR engagement can lead to redistribution of cytoskeletal elements such as talin, vinculin, and actin (9, 24). The cross-linking Ab might be providing sufficient TCR aggregation to trigger a threshold signal for cytoskeletal mobilization. Studies by Valetutti et al. (25) have suggested that the cytoskeleton also plays an active role in sustaining a TCR signal, because the addition of agents which disrupt the actin cytoskeleton (e.g., Cytochalasin D) can block the rise in intracellular Ca $^{2+}$ . The cross-linked anti-CD3 system may be useful for dissecting the role of the cytoskeleton in proximal signaling events.

Our experiments suggest that efficient recruitment of lck may be the pivotal event accomplished by aggregation. Lck has been shown to be important for proximal signaling in that absence of lck almost completely abrogates tyrosine phosphorylation events (9, 31). It is well established that coaggregating anti-CD3 and anti-CD4 antibodies or using anti-CD3/anti-CD4 heteroconjugate mAbs can result in enhanced tyrosine phosphorylation and calcium mobilization (32). Recently, it was shown that in circumstances in which lck is limiting, as in double-positive thymocytes,

ZAP-70 phosphorylation requires coaggregation of TCR and CD4 (28). Furthermore, blocking CD4 (and presumably its associated lck molecules) with anti-CD4 mAbs can convert a partial agonist signal into an antagonist signal with its associated characteristic signaling deficits (33). Thus, impaired CD4 recruitment has been proposed as a mechanism for altered peptide/antagonist peptide signaling. The pivotal nature of lck recruitment is underscored by our finding that secondary antibody induced aggregation can be dispensed with, if lck is recruited by bringing CD4 into the complex artificially. Even in the absence of exogenous cross-linking, stimulation with a bivalent anti-CD3  $\times$  anti-CD4 reagent reconstituted both the early signaling events of ZAP-70 phosphorylation and association of other phosphorylated proteins with the complex. In turn, these early events lead ultimately to a mitogenic stimulus.

The partial signals delivered by nonmitogenic anti-CD3 correlated with the induction of functional anergy as defined by an inability to proliferate due to poor IL-2 production. The striking similarity between the signals delivered by altered peptide ligands and nonmitogenic anti-CD3 are perhaps indicative of a common mechanism of anergy induction. How these partial signals translate into an off signal that shuts down T cell clonal responsiveness has yet to be determined. In the classical model of anergy, involving a complete signal one (through the TCR) in the absence of signal two (costimulation), induction of unresponsiveness depends upon a successful calcium signal that can be blocked by CsA (34, 35). Similarly, CsA has been shown to block anergy induction by altered peptide ligands (12). In fact, an altered peptide ligand triggered calcium signal has been recently demonstrated using an exquisitely

sensitive system (36). The ability of CsA to block nonmitogenic anti-CD3-induced functional anergy suggests that a calcium signal may be important in this process. It is possible that the lack of detectable calcium flux by nonmitogenic anti-CD3 reflects insufficient sensitivity. In contrast with the classical models of anergy, the presence of competent APC or anti-CD28 antibodies did not rescue T cell clones from nonmitogenic anti-CD3-induced unresponsiveness.

An unresolved observation was the finding that culture with the nonmitogenic anti-CD3 suppresses IL-2 production in clones, but it did not appear to impair significantly the responsiveness of bulk T cells. Our results suggest similar defects in signaling between naive cells and clones in terms of ZAP-70 phosphorylation and TCR-CD3 complex-associated phosphorylated molecules, as well as the downstream events of PLC $\gamma$ -1 phosphorylation and TCR capping. It is possible that naive cells and clones differ in the way they respond to nonmitogenic anti-CD3 mAbs, either in the triggering of other biochemical signals, or the integration of downstream nuclear signals.

The mitogenic forms of anti-CD3 currently in use severely suppress global T cell responses. This study suggests that nonmitogenic anti-CD3 may selectively induce unresponsiveness in activated T cell subsets. These findings bear promising implications for transplant therapy in that it would be beneficial to be able to suppress the alloreactive T cells that mediate graft rejection, while maintaining the responsiveness of other T cells. Further investigation into the mechanisms by which nonmitogenic anti-CD3 modulates T cell responses should shed some light on the applicability of this potential therapeutic to transplantation.

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